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TITLE: Targeted Therapies for Myeloma and Metastatic Bone Cancers

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San Antonio TX 78238

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

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- Quantified functional groups available for ligand conjugation using S_{35}-labeled ligands.
- Developed alternative assay to confirm affinity of bone-targeting nanoparticles to hydroxyapatite substrates.
- Demonstrated in vitro stability of radiolabeled nanoparticles.
- Successfully transferred our nanoparticle preparation protocols to another facility to support in vivo biodistribution studies.
- Started in vivo biodistributions, validating ability of our radiolabeled nanoparticles to be imaged for up to 48 hours and further confirming our protocol methods to study bone-targeting nanoparticle biodistribution via radio-imaging.

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Table of Contents

Introduction

The goal of this project is to determine, in preclinical studies, the potential of skeletally targeted proteasome inhibitors as an efficacious and selective treatment for myeloma. We have found that several proteasome inhibitors are effective against both human and murine myeloma cells in culture. However, as with any proteasome inhibitor, there are serious concerns over the potential systemic effects and toxicity. Our hypothesis is that bone-targeting nanocarriers can preferentially accumulate in the skeleton and locally release proteasome inhibitors to impair the capacity of myeloma cells to survive and grow in vivo, thereby reducing the formation and growth of tumor-induced lytic bone lesions. Proteasome inhibitors are otherwise not selective to bone and their therapeutic-toxic window may be narrow when administered systemically. The scope of this project is to validate our hypothesis. The major tasks are: 1. Formulate and characterize drug-containing, bone-targeting nanocapsules; 2. Determine the in vivo biodistribution of bone-targeting nanocapsules; and, 3. Evaluate the efficacy of bone-targeted delivery of proteasome inhibitors on myeloma tumor progression using the 5TGM1 murine model of myeloma. The outcomes of this research will be significant. The study will demonstrate the preferential biodistribution of nanocarriers specifically designed to target and adhere to bone matrices. It will further show that these same nanocapsules can selectively deliver a specific and potent proteasome inhibitor to skeletal sites to act as an anti-myeloma agent. Targeted bone delivery has several potential benefits, including reduced systemic exposure, increased efficacy in the targeted microenvironment, and the ultimate opportunity to reverse catastrophic disease processes. Furthermore, targeted delivery to bone has several additional significant application opportunities in the areas of osteoporosis, fracture healing, cartilage repair, and tissue engineering.

Body

The project is broken down into the following tasks:

1. Formulate and characterize drug-containing, bone-targeting nanocarriers
2. Determine the in vivo biodistribution of bone-targeting nanocarriers
3. Demonstrate the efficacy of bone-targeted delivery of proteasome inhibitors on myeloma tumor progress

Task 1 was scheduled to occur during years 1 – 2 of the project, Task 2 during years 2 – 3 of the project with some overlap with Task 1, and Task 3 is scheduled to occur during the last year and one-half of the project.

Task 1 is focused on the development of the bone-targeting nanoparticles and is broken down into the following subtasks:

1. Selection of proteasome inhibitors for in vivo studies – Completed and reported in first annual report.
2. Formulation and characterization of bone-targeting nanoparticles – Completed and discussed in first and second annual reports.

3. Demonstration of adhesion of bone-targeting nanoparticles to bone-like substrates in vitro – Preliminarily discussed in second annual report and discussed further in this annual report.
4. Formulation of proteasome inhibitors into bone-targeting nanoparticles – Preliminarily discussed in second annual report. Additional work postponed until completion of biodistribution studies discussed in Task 2.

The bulk of the work during this year of the project was focused on further development and characterization of bone-targeting nanoparticles. This consisted of the following tasks:

1. Radiolabeling of nanoparticles
2. Quantifying ligand attachment to nanoparticles
3. In vitro binding of ligated nanoparticles to bone-like surfaces

We developed methods of radiolabeling nanoparticles (Task 2.1, Proposal Section 5.5.1) in preparation for biodistribution studies planned in Task 2. We selected the gamma emitter ^{99m}Tc, with a 6.5hr half-life, based on the experience of our collaborators at the University of Texas Health Science Center-San Antonio (UTHSC). ^{99m}Tc is hydrophilic and provided as an aqueous solution from the cyclotron source. We originally proposed chelating this radionuclide with a lipophilic chelator, mirroring methods to label liposomes, to facilitate encapsulation using our simple precipitation protocol. This did not work. After examining several commercial chelators, we found both the chelation and the encapsulation efficiency to be very dependent on the 'quality' of the radionuclide. ^{99m}Tc is subject to oxidation, which affects chelation efficiency, which in turn affects encapsulation efficiency. In most cases, encapsulation efficiency was less than about 20%, which we deemed insufficient. Subsequently, we explored the conjugation of ^{99m}Tc to reduced proteins, such as bovine serum albumin (BSA), to improve the loading efficiency into nanoparticles. This yielded encapsulation efficiencies of 90% or greater (see Figure 2). Payload stability was monitored over a 24-hour period and was found to be nearly 100%. However, we had concerns about residual reducing agent used to activate the protein interfering with coupling of bone-targeting ligands to functionalized nanoparticles. Therefore, we modified the radiolabeling method to use a commercial reducing gel that can be removed from the protein preparation by simple centrifugation. The newly reduced, clean protein can then be used to complex the radionuclide for encapsulation using the same procedure as before. The encapsulation efficiency is unaffected by this slightly altered approach. Furthermore, this modified method avoids a lengthy column separation process that diminishes the amount of available radioactivity.

Quantification of bone-targeting ligand content on nanoparticle surfaces has been the most difficult aspect of this work to date. The issue is simply that the amount of functional activity on the surface of nanoparticles, either in the form of a functional group (i.e. maleimide) or as attached ligand (e.g. amino bisphosphonate or aspartic acid oligomers), is extremely small, so very sensitive methods are required. Interestingly, this is something that is not addressed in the current literature. Commonly, the existence of targeting ligands is indirectly assessed by the ability of the attached particle to achieve

targeting and this is currently how we are assessing the presence of targeting ligands. In our approach, we are conjugating ligands to preformed nanoparticles containing functionalized surfaces and incubating them with hydroxyapatite powders. The nanoparticles are radiolabeled to increase the sensitivity of the assay and we have shown preferential adhesion of ligand-containing nanoparticles to hydroxyapatite substrates in vitro.

The above approach is an indirect measure of the existence of the bone-targeting ligands. We continued to explore direct methods to quantitate ligand attachment. In one approach we used a radiolabeled ligand surrogate to ascertain the presence of free functional groups for ligand attachment. Using radioactive cysteine (^{35}S) we showed functional groups for ligand attachment were present on formulated nanoparticles. In another approach, we showed via a fluorescent assay that one of our ligands, Asp_4, an oligopeptide, was bound to the nanoparticle surface and this was a function of available functional groups for ligand attachment. These particles were shown to adhere preferentially to hydroxyapatite substrates in vitro.

Task 2 is concerned with determining the in vivo biodistribution of bone-targeting nanoparticles in myeloma mouse model. The task is broken down into the following subtasks:

1. Prepare radiolabeled bone-targeting nanoparticles – Completed.
2. Conduct in vivo biodistribution assay.

Task 2 has been delayed by operational and technical difficulties. The task was initially delayed by a change of venue of our collaborator, Dr. Mundy, who was originally with the University of Texas Health Science Center at San Antonio (UTHSC-SA) and has now moved to new facilities at Vanderbilt University. Dr. Mundy's operations were down until early Summer '07, by which time it was too late to consider starting in vivo animal studies, since the required animals come from suppliers in Europe and shipments are not generally made during the hot Summer months to avoid animal loss. We were not able to schedule studies until Nov. 07 due to noted shipping restrictions and an imaging equipment upgrades conducted in early Fall by the imaging facility. We conducted initial animal trials in late Nov/early Dec, which allowed us to confirm our in vivo protocols and test initial nanoparticle formulations. Particle formulations and radiolabel contents were more than sufficient to permit in vivo imaging; however, the initial particle size of 180nm proved too large to support long-term distribution. This led to further development to reduce the particle size, which raised some previously unknown issues with nanoparticle formulation by-products on smaller nanoparticle colloidal stability. This forced use to resolve these issues before proceeding with additional in vivo studies. These technical difficulties have been solved, but we have been further delayed by a nationwide shortage of Tc^{99m}, which started in late Dec '07 and has only recently resolved itself. We now have new studies planned for late February, to be followed up with additional studies in late March. We plan to have in vivo biodistribution studies completed by late Spring.

Task 3 is concerned with demonstrating the efficacy of bone-targeting nanoparticles containing small molecule therapies in a myeloma mouse model. The task is broken down into the following subtasks:

3. Prepare radiolabeled bone-targeting nanoparticles – pending.
4. Conduct in vivo efficacy studies – pending.

Key Research Accomplishments

- Developed methods to radiolabel polymer nanoparticles, the first time, to our knowledge, this has been done.
- Quantified functional groups available for ligand conjugation using S^{35}-labeled ligands.
- Developed alternative assay to confirm affinity of bone-targeting nanoparticles to hydroxyapatite substrates.
- Demonstrated in vitro stability of radiolabeled nanoparticles.
- Successfully transferred our nanoparticle preparation protocols to another facility to support in vivo biodistribution studies.
- Started in vivo biodistributions, validating ability of our radiolabeled nanoparticles to be imaged for up to 48 hours and further confirming our protocol methods to study bone-targeting nanoparticle biodistribution via radio-imaging.

Reportable Outcomes

- Manuscript in preparation discussing the development of bone-targeting nanoparticles and radiolabeled nanoparticles.
- Presented poster at 32nd Annual Meeting of the Society of Biomaterials.
- Made podium presentation entitled "Development and Characterization of Bone-Targeting Nanoparticles" at the 34th Annual Meeting of the Controlled Release Society, July 7 – 11, Long Beach, CA.
- Presented poster entitled "Controlled Release of Proteasome Inhibitor from Biodegradable Nanoparticles for Myeloma Therapies" at the 34th Annual Meeting of the Controlled Release Society, July 7 – 11, Long Beach, CA.
- Hired additional professional staff to support biodistribution and in vivo efficacy studies.
- Animal protocols for the biodistribution and efficacy studies described in the original statement of work (Tasks 2 & 3, respectively) have been reviewed and approved by the DoD Animal Care & Use Review Office.

Conclusions

The completed work positions the project to continue biodistribution studies to study the performance of bone-targeting nanoparticles in vivo. We can consistently prepare polymer nanoparticles of required size and composition necessary to support other tasks of the project. Technical difficulties encountered during the development of the bone-

targeting nanoparticles, the relocation of our collaborator, Dr. Mundy, and interruptions in the supply of key materials has delayed the project by more than one year, such that Task 2 remains to be completed, but is expected to be completed by late Spring, and Task 3 has not started.

References

None.

Appendix 1
12th Quarterly Report

Quarterly Report 12

. Contract No. W81XWH-05-C-0004 2. Report Date 15 February 2008

3. Reporting period from 1 November 2007 to 31 January 2008

4. PI Neal Vail, Ph.D. 5. Telephone No. 210 522 5351

6. Institution Southwest Research Institute

7. Project Title Targeted Therapies for Myeloma and Metastatic Bone Cancers

8. Current staff, with percent effort of each on project.

PL3	4%	Senior Tech	<1%
PL1	59%	Clerical	<1%

9. Contract expenditures to date (as applicable):

	This Qtr / Cumulative		This Qtr / Cumulative
Personnel		Travel	
Fringe Benefits		Equipment	
Supplies	Other		

	This Qtr / Cumulative
Subtotal	
Indirect Costs	
Fee	
Total	

10. Comments on administrative and logistical matters.

There have been no administrative or logistical problems encountered as yet.

11. Use additional pages as necessary to describe scientific progress for the quarter in terms of the tasks or objectives listed in the statement of work for this contract.

See attached pages.

12. Use additional pages to present a brief statement of plans or milestones for the next quarter. We will complete biodistribution studies during the next quarter. We will be conducting further drug encapsulation studies and examining their release profiles and drug activity, all in preparation for efficacy studies later next year.

Project Overview

Multiple myeloma is the second most common adult hematologic malignancy. The disease is uniformly fatal with 80% of patients suffering devastating and progressive bone destruction. SwRI has teamed with the University of Texas Health Science Center at San Antonio, OsteoScreen, and Walter Reed Army Medical Center to develop and evaluate bone-targeting nanocarriers that can preferentially accumulate in the skeleton and local release proteasome inhibitors, such as MG-262, to impair the capacity of myeloma cells to survive in vivo, thereby reducing the formation and growth of tumor-induced lytic bone lesions.

The following tasks were investigated during the recent reporting period:

1. Quantification of Ligand Content on Nanoparticles
2. Nanoparticle Size Reduction for In Vivo Studies

Each of these items is discussed in more detail in the following sections.

Ligand Content of Nanoparticles (Task 1.3, Proposal Section 5.3)

We developed a new method to determine the conjugation efficiency of bone-targeting ligands to our nanoparticles. We proposed studying two ligands: amino bisphosphonate and an aspartic acid oligomer (Asp_4). We have been working exclusively with Asp4 due to the simpler chemistry of this ligand relative to the bisphosphonate; however, the methods developed herein can be translated to the bisphosphonate when we consider this ligand. The difficulty in determine the ligand conjugation efficiency to nanoparticles is the relatively low quantities involved relative to the overall system. Therefore, we have focused on the Asp_4 due to its larger molecular size and larger opportunity for detection. We conjugate both ligands by reducing their amino terminus to a thiol by reaction with imino-thiolane (Traut's Reagent) followed by coupling to nanoparticles containing block copolymers of PLA-PEG with maleimide (MAL) terminus on the PEG block. We reported the synthesis and characterization of these functional block copolymers in previous reports.

The amount MAL was varied at three levels (80:20; 90:10; 96:4 – w/w) by mixing different proportions of the function copolymer with unmodified PLGA polymer. A 10-fold excess of $(Asp)_4$ peptide ligand was reduced and incubated with freshly prepared nanoparticles at room temperature overnight. The nanoparticles were separated from unconjugated peptide by ultracentrifugation and the pellet dispersed in 1 ml of DI water. The nanoparticle dispersion was extensively hydrolyzed overnight using constant boiling HCl at 120 °C. After hydrolysis, the resulting solution was neutralized and assayed for released aspartate (Asp) amino acid.

A *o*-Phthaldialdehyde (OPA) assay was used to quantify the amount of amino acids. It has been well established that binding of amino acids to OPA compound causes an increase in intrinsic fluorescence of OPA compound. To quantify the amount of released

amino acids we developed a standard calibration curve by incubation varying concentration of Asp amino acid with OPA solution. The representative calibration curve for Asp amino acid quantification is shown below (Figure 1). The fluorescence intensity was normalized for the background fluorescence of OPA compound.

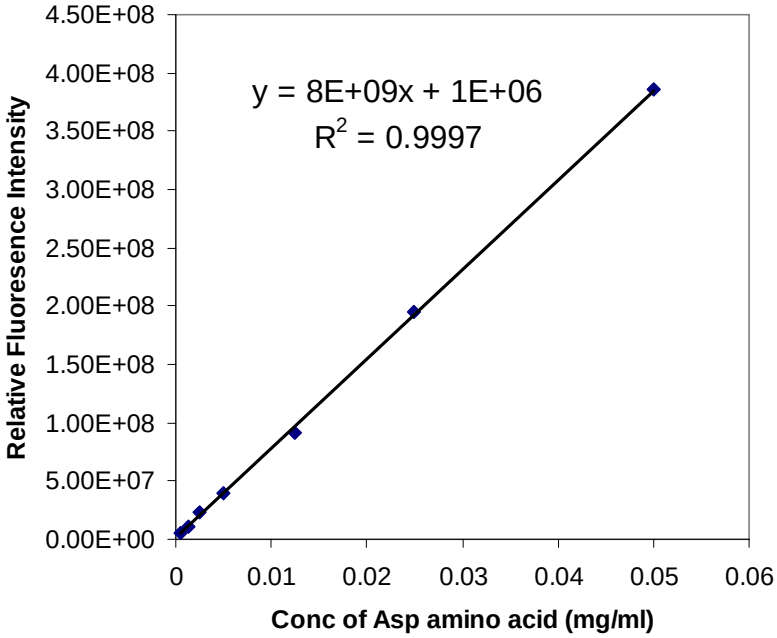

Figure 1. Calibration curve for OPA assay of amino acid content.

Nanoparticles without conjugated Asp_4 ligand were controls to determine the increase in OPA fluorescence due to any non-specific interaction with polymer fractions. The contribution of control samples was subtracted to determine the effective concentration of hydrolyzed peptide. Figure 2 shows the resultant concentration of Asp peptide conjugated to nanoparticles at varying levels of MAL concentration in the nanoparticles.

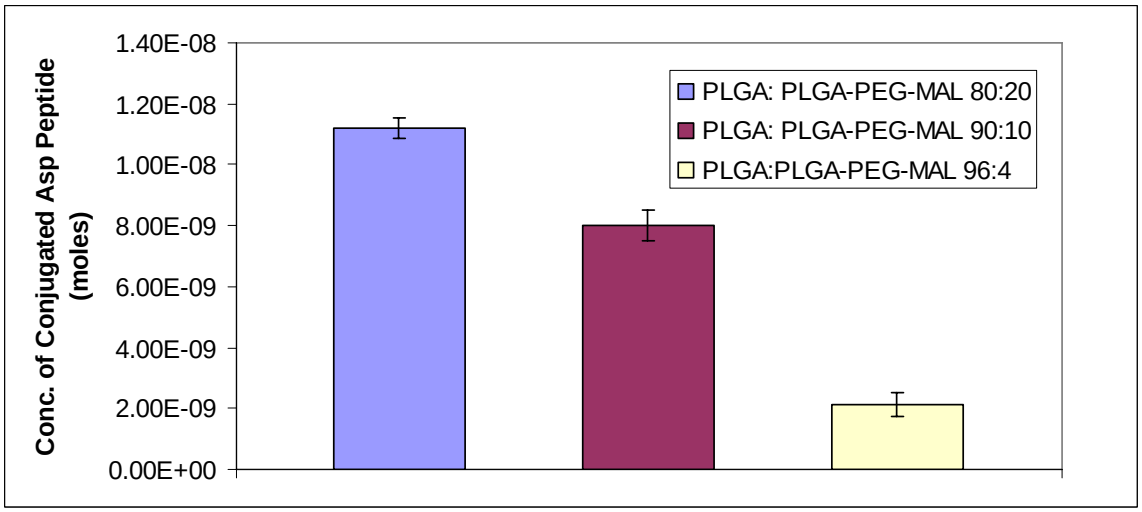

Figure 2. Amounts of conjugated Asp_4 ligand to nanoparticles containing different compositions of ligand binding sites.

The results show that the amount of conjugated Asp_4 ligand follows the availability of MAL functional groups over the given compositional range. The results also highlight that the conjugation efficiency is low. For 80:20 PLGA nanoparticles, the efficiency was approximately 3%. Low conjugation efficiency can be attributed to various factors including accessibility of Mal molecule for conjugation, rapid hydrolysis Maleimide group (~half life of 30-40 minutes), steric hindrance for Asp peptide molecule and possibly a low efficiency in incorporation of polymer chains to form nanoparticles. To obtain a more accurate measure of conjugation efficiency, we need to determine the concentration of nanoparticles formed using the above approach.

Nanoparticle Size Reduction for In Vivo Studies (Task 2.1, Proposal Section 5.5.2)

In our last report we discussed our initial in vivo biodistribution trials with nanoparticles developed during this work. We developed methods to prepare radiolabeled particles and characterized their encapsulation efficiency and stability. These initial particles typically had particle sizes on order of about 180nm. Our initial in vivo studies confirmed this size is too larger resulting in rapid clearing of the nanoparticles by the liver. Therefore, we have been refining our nanoparticle preparation procedure to produce particles on the order of 110 – 130nm, which are expected to have better circulation properties.

We have been successful in preparing particles with appropriate size, but have encountered some issues with preparation by-products have deleterious effects on the colloidal stability of these smaller nanoparticles. Specifically, we have found that by-products from the conjugation of Tc^{99m} to BSA, such residual reducing agent ($NaBH_4$) and activator (stannous chloride) can lead to nanoparticle aggregation. We have overcome these issues by reducing the reactant amounts and introducing an additional column cleanup procedure. This improved stability, but long-term stability (e.g. > 1hr) remained an issue. We subsequently found that non-encapsulated BSA was the source of this instability. We found that digesting non-encapsulated BSA with trypsin followed by a centrifuge cleanup restored long-term nanoparticle stability.

We have reduced the nanoparticle size to ~120nm while maintaining high radiolabel encapsulation efficiency and long-term particle stability. We have schedule new sets of animal studies to examine the in vivo performance of these particles. Scheduling these new studies has been delayed due to a nationwide shortage of Tc^{99m} that started in early December, 2007, and is only now beginning to return to normal.

Next Steps

We will complete biodistribution studies during the next quarter. We will be conducting further drug encapsulation studies and examining their release profiles and drug activity, all in preparation for efficacy studies later next year.